Fine-tuning and autoregulation of the intestinal determinant and tumor suppressor homeobox gene *CDX2* by alternative splicing

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On the basis of phylogenetic analyses, we uncovered a variant of the *CDX2* homeobox gene, a major regulator of the development and homeostasis of the gut epithelium, also involved in cancer. This variant, mini*CDX2*, is generated by alternative splicing coupled to alternative translation initiation, and contains the DNA-binding homeodomain but is devoid of transactivation domain. It is predominantly expressed in crypt cells, whereas the *CDX2* protein is present in crypt cells but also in differentiated villous cells. Functional studies revealed a dominant-negative effect exerted by mini*CDX2* on the transcriptional activity of *CDX2*, and conversely similar effects regarding several transcription-independent functions of *CDX2*. In addition, a regulatory role played by the *CDX2* and mini*CDX2* homeoproteins on their pre-mRNA splicing is displayed, through interactions with splicing factors. Overexpression of mini*CDX2* in the duodenal Brunner glands leads to the expansion of the territory of these glands and ultimately to brunneroma. As a whole, this study characterized a new and original variant of the *CDX2* homeobox gene. The production of this variant represents not only a novel level of regulation of this gene, but also a novel way to fine-tune its biological activity through the versatile functions exerted by the truncated variant compared to the full-length homeoprotein. This study highlights the relevance of generating protein diversity through alternative splicing in the gut and its diseases.

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The intestinal epithelium is a complex cellular system in constant renewal whose dynamic homeostasis involves multiple and complementary factors and pathways.^{1,2} Among them, the homeotic transcription factor *CDX2* has a central role during embryogenesis to determine the intestinal identity of the presumptive midgut/hindgut endoderm,^{3,4} and throughout adulthood for intestinal epithelium homeostasis to organize the stem cell niche, to provide tissue identity to the stem cells, and to regulate cell proliferation and differentiation.^{5–8} *CDX2* gene expression and function need to be tightly regulated, because both loss and overexpression are lethal.^{8,9} *CDX2* is also relevant in gut pathologies, since its strong decay in human colon cancer correlates with poor evolution,^{10,11} while functional studies have attributed a tumor suppressor role in the gut.^{12–15}

Alternative splicing of pre-messenger RNA is an important facet of RNA metabolism to generate protein diversity^{16,17} involved in every biological process from embryonic development to tissue homeostasis, and also in diseases.^{18,19} However, it remains largely underrated because most of the splicing variants are only identified by their sequence without knowledge on their function. Alternative splicing is poorly

documented in the gut. Here, we discovered a splicing variant of *CDX2* and we unveiled its original function.

Results

The *CDX2* homeobox gene encodes an alternative mRNA variant, mini*CDX2*. To search for novel transcript isoforms in the mouse intestine, 490 million paired-end reads were generated from three independent RNA samples of the cecum. They corresponded to 16 822 consistently expressed genes (>10 normalized reads in all replicates), and represented 43% of all annotated genes in the mouse genome including, for instance, 98% of the intestinal stem cells markers.²⁰ The comparison to the reference transcripts annotation identified 4799 novel possible exon–exon junctions in 1802 genes (Supplementary Table 1). This highlights the potential of alternative splicing to generate protein diversity in the gut.

Among those genes is *CDX2*, a homeobox gene containing three exons (Figure 1A). Exploring its functional structure by RT-PCR and northern blot in human colon cancer cell lines and mouse intestine identified the variant referred to as

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Figure 1 Alternative splicing at the *CDX2* locus. (A) *CDX2* gene map. E1–3: exon-1 to -3; I1 and 2: intron-1 and -2; dotted lines represent the spliced regions to produce, respectively, the *CDX2* and *miniCDX2* mRNAs. The translation start codons ATG₁ and ATG₂, and the stop codons Stop₁ and Stop₂ are indicated. (B) Expression of the *miniCDX2* transcript. (a) RT-PCR on intestinal cell lines with the primers CDX21F/CCR hybridizing, respectively, upstream of ATG₁ in the exon-1 and downstream of Stop₂ in the exon-3. The two bands correspond to the *CDX2* and *miniCDX2* transcripts. (b) Northern blot of polyA RNA (10 μ g per lane) from Caco2TC7 cells cultured for 3 days and 14 days revealing the *CDX2* and *miniCDX2* mRNAs. (c) RT-qPCR quantification of *CDX2* (light gray) and *miniCDX2* mRNA (dark gray) in 3 days Caco2TC7 cells (semi-logarithmic scale). (d) RT-qPCR of *CDX2* (light gray) and *miniCDX2* mRNA (dark gray) along the murine gut (semi-logarithmic scale); *CDX2* was arbitrary set at 1 in the cecum. (e) *CDX2* / *miniCDX2* mRNA ratio along the mouse intestine

miniCDX2 (Figure 1B). The level of this variant was far below that of the *CDX2* mRNA (15-times less abundant) in proliferating Caco2TC7 cells (3 days in culture) and further decreased in differentiated cells (14 days in culture). The *miniCDX2* mRNA was up to 20-times less abundant than the *CDX2* mRNA along the murine intestine.

MiniCDX2 corresponds to an evolutionary-conserved alternative splicing variant. The production of the *miniCDX2* variant involves a canonical GU splicing donor site within the exon-1, instead of the standard donor site at the end of the exon used to produce the *CDX2* mRNA (Supplementary Figure S1). Remarkably, the alternative donor site overlaps the translation initiation codon of the *CDX2* protein in the *CDX2* mRNA (referred thereafter to as AUG₂) that is therefore destroyed in the *miniCDX2* transcript. Yet, the *CDX2* gene sequence contains one additional AUG located 29 pb upstream of AUG₂. This AUG, referred to as AUG₁, is out-of-frame with AUG₂ but in-frame with the open reading frame of exon-2 and -3 in the *miniCDX2* transcript. AUG₁ is preceded by GGCAGC and AUG₂ by GCCACC, both being consistent with a consensus Kozak box (GNCANCAUG).²¹ The *CDX2* mRNA, but not the *miniCDX2* variant, additionally contains a translation stop codon (Stop1) located four nucleotides downstream of AUG₂, thus in-frame with AUG₁.

Strikingly, the motif composed of (i) the GU splicing donor site overlapping the AUG₂ start codon of the *CDX2* protein, (ii) the putative upstream start site AUG₁ and (iii) the stop codon Stop1 in-frame with AUG₁, is evolutionary-conserved in vertebrates from coelacanth to human, including marsupials, birds, reptiles, amphibians, the bichir (the most basal extant ray-finned fish) and the bowfin (the immediate outgroup to teleost fish) (Figure 2A; Supplementary Figure S2). However, it is found neither in *CDX1*-type genes (except for *Cdx1b* in teleost fishes like *Danio rerio*, see 'Discussion'), nor in *CDX4*-type genes.

The *miniCDX2* variant encodes a truncated form of the *CDX2* homeoprotein. To study the translational activity of the upstream AUG₁ and to compare it with AUG₂ used for *CDX2* translation, reporter plasmids were constructed in which the 5'-untranslated sequence of the *CDX2* gene ending at ATG₂ (pATG1-ATG2) or ATG₁ (pATG1) was linked to the Luciferase coding sequence (Figure 2Ba). The translation activity of AUG₂ used for *CDX2* synthesis was verified by the production of Luciferase in HCT116 cells transfected with pATG1-ATG2 that recapitulates the configuration of the *CDX2* mRNA in which AUG₂ is in-frame and AUG₁ out-of-frame with the reporter sequence. Interestingly, pATG1 ending at ATG₁ and recapitulating the *miniCDX2* configuration produced an even higher luciferase activity than pATG1-ATG2. This activity was abolished by mutating ATG₁

Figure 2 Functionality of the conserved alternative splicing/translation motif of the *CDX2* locus. (A) The conserved motif found in *CDX2*-type genes (see also the Supplementary Figure 2). (B) (a) Luciferase activity in human colon cancer HCT116 cells transfected with the indicated reporter plasmids; X designates the mutation of ATG₁ into TAG in pATG1m and in pATG1m-ATG2; pGI3-control was set to 100 and pGI3-basic gave background activity. (b) Luciferase activity in HCT116 cells transfected with *in vitro*-transcribed polyadenylated RNA without (light gray) or with m7G cap (dark gray). Data are given as mean $\pm$ S.D. for triplicates

into TAG in pATG1m, while changing ATG₁ into TAG in pATG1m-ATG2 increased luciferase compared to pATG1-ATG2. Thus, AUG₁ is translationally active and, in the context of the *CDX2* mRNA, it partially hinders the translational activity of AUG₂.

In the *CDX2* mRNA, the AUG₂ start codon used for the CDX2 protein is preceded by the translationally competent AUG₁. Ribosomes generally start translation at the first AUG after cap-dependent scanning of the 5'-untranslated region, but can skip it for a second site by leaky scanning²² or reach an internal codon using a cap-independent internal ribosome entry site.²³ To compare the cap-dependency of AUG₁ and AUG₂, uncapped and m7G-capped reporter RNAs named RNA_{AUG1} and RNA_{AUG1-AUG2} were *in vitro* transcribed from PCR templates derived from pATG1 and pATG1-ATG2, respectively, and transfected in HCT116 cells. Luciferase activity was higher for capped *versus* uncapped RNA_{AUG1}, and for capped *versus* uncapped RNA_{AUG1-AUG2}. Moreover, destroying AUG₁ in RNA_{AUG1m-AUG2} increased luciferase compared to RNA_{AUG1-AUG2} (Figure 2Bb). Thus, the activity of AUG₂, like AUG₁, is cap-dependent, suggesting that ribosomes reach AUG₂ for CDX2 translation by leaky scanning through AUG₁.

The sequence of the splicing mRNA variant predicts the production of a CDX2-related homeoprotein, miniCDX2, in which a 12-aa N-terminus segment replaces the 181-aa transactivation domain (Figure 3A, Supplementary Figure S1). An antibody, C2T, was raised against this 12-aa segment and validated using expression plasmids (Figure 3Ba), peptide competition and conditional *CDX2* knockout mice (Figure 3Bc). Consistent with the presence of the DNA-binding homeodomain, the miniCDX2 protein produced by pFlag-miniCDX2-transfected HCT116 cells was nuclear (Figure 3Bb). Compared to CDX2 present in almost every intestinal crypt and villous epithelial cell from embryos to adults, miniCDX2 was not detected in the embryonic gut, it appeared perinatally at the level of the intervillous epithelium at the origin of the crypts (Figure 3Ca), and remained expressed throughout adult life in the small intestinal crypts and in the bottom of the colonic glands (Figures 3Ca and c). Crypt cells co-expressed miniCDX2 and CDX2 (Figure 3Cb).

In small intestinal tumors of *Apc*^{D14/+} mice, CDX2 expression is known to become reduced and heterogeneous. Interestingly, miniCDX2 was also expressed heterogeneously and in the same tumor regions as CDX2 (Figure 3Da). Moreover, RT-qPCR showed that the ratio between the *CDX2*

and *miniCDX2* transcripts decreased in these tumors compared to the normal mucosa ($P < 0.05$) (Figures 3Db and c).

Opposite and similar molecular functions of miniCDX2 compared to CDX2. We next addressed the molecular functions of miniCDX2 compared to CDX2. As a transcription factor, the CDX2 protein activates a large number of downstream genes including the sucrase–isomaltase (*SI*) gene. In HCT116 cells transfected with pFlag-CDX2 or pFlag-miniCDX2, the CDX2 protein, but not miniCDX2, stimulated the level of *SI* mRNA and the activity of the *SI* gene promoter assayed using the *SI* luciferase reporter; moreover, miniCDX2 dose-dependently abolished the stimulatory effect of CDX2 (Figures 4Aa and b). Chromatin immunoprecipitation (ChIP) revealed the ability of miniCDX2 to bind the same chromatin targets as CDX2, for example, the *SI*, *Li-cadherin* and *Muc2* promoters (Figure 4Ac), and to reduce the binding of CDX2 to the *SI* promoter (Figure 4Ad). Thus, miniCDX2 is transcriptionally inefficient, but acts as a dominant-negative regulator of the transcriptional activity of CDX2 by DNA-binding competition.

CDX2 exerts broader functions than regulating transcription through DNA binding.²⁴ For instance, it can bind β -catenin to prevent its interaction with Tcf4, thus impeding Wnt/ β -catenin signaling.²⁵ Indeed, CDX2 opposed the stimulation of the *Mmp7* mRNA, a Wnt target, and the activity of the TOP-Flash Wnt reporter by β -catenin/Tcf4 (Figures 4Ba and b). Unlike CDX2, miniCDX2 failed to interfere with the β -catenin/Tcf4 stimulation of *Mmp7* mRNA and TOP-Flash activity. In addition, it failed to relieve the inhibitory effect of CDX2 on β -catenin/Tcf4 (Figures 4Ba and b), and did neither interact with β -catenin nor prevent β -catenin/Tcf4 interaction (Figures 4Bc and d). Thus, contrary to its dominant-negative effect on the CDX2 transcriptional activity, miniCDX2 is neutral with respect to the inhibitory function exerted by CDX2 on β -catenin/Tcf4 signaling.

CDX2 also exerts transcription-independent functions including the increase of P27^{KIP1} to compromise cell proliferation.²⁶ Like CDX2, miniCDX2 increased the level of P27^{KIP1} protein without changing its mRNA (Figures 4Ca and b) and it reduced cell growth in a colony formation assay (Figure 4Cc). The CDX2 protein has also been reported to inhibit double-stranded DNA breaks repair in HCT116 cells by interacting with the KU70/80 complex.²⁷ Here, we extended these observations by showing in SW480 colon cancer cells

that miniCDX2, like CDX2, also co-immunoprecipitated with KU70/80 proteins, and hampered DNA repair activity (Figures 4Da and b).

Thus, miniCDX2 fine-tunes the activity of CDX2 in that it exerts similar and/or different effects with respect to the multifaceted functions of the full-length homeoprotein: it is dominant-negative on the transcriptional activity of CDX2, it is neutral as regards the inhibitory role of CDX2 on β -catenin/Tcf4 interaction, and it exerts similar transcription-independent effects as CDX2 on cell growth inhibition by P27^{KIP1} and on double-stranded DNA break repair through KU70/80.

MiniCDX2 participates in the control of intestinal homeostasis. We next explored the role of miniCDX2 *in vivo* in mice using a gain-of-function approach. For this purpose, transgenic *jojo-Flag-miniCDX2* mice were generated, allowing inducible expression of the Flag-miniCDX2 protein (Figure 5A). Crossing *jojo-Flag-miniCDX2* and *Sox2Cre* mice to broadly express Flag-miniCDX2 in the zygote caused embryonic death, which is reminiscent of the lethality resulting from *CDX2* knockout (0 living double-transgenics over 80 descendants). In the adult gut of *jojo-Flag-miniCDX2* mice (three independent founders), GFP immunostaining used to trace the activity of the CAG promoter revealed only very few villi with transgene expression ($< 1\%$, Supplementary Figure S3Ba). Consequently, *jojo-Flag-miniCDX2::VilCre* mice generated to target the intestinal epithelium showed only rare villi with Flag-miniCDX2 expression. In those villi with a sustained expression of Flag-miniCDX2, intestinal alkaline phosphatase was lost, whereas gastric Claudin-18 was turned on (Figure 5B), without apparent change in CDX2. This reproduced the differentiation switch induced by targeted *CDX2* knockout in the gut,²⁸ indicating that miniCDX2 is able to antagonize the transcriptional potential of CDX2 *in vivo*.

Brunner's glands (BGs) are glandular structures connected to the crypts of the floor of the proximal duodenum. They express both CDX2 and miniCDX2 at low levels (not shown). Interestingly, the CAG promoter is active in the BGs of *jojo-Flag-miniCDX2* mice, as revealed by GFP immunostaining, thus allowing a widespread expression of Flag-miniCDX2 in the BGs of *jojo-Flag-miniCDX2::VilCre* mice (Supplementary Figures S3Bb and C). Noteworthy, these mice exhibited a 2.5-fold enlargement of the territory of the BGs (Figure 5C).

Figure 3 Protein expression of the miniCDX2 variant. (A) Structure of the CDX2 and miniCDX2 proteins: TA, transactivation domain; HD, DNA-binding homeodomain; R, regulatory domain. The arrow points to the 12-aa Nter peptide of miniCDX2. (B) The C2T antibody raised against the 12-aa Nter peptide of miniCDX2. (a) HCT116 cells transfected with the plasmid pFlag-CDX2 (lane 1), pFlag-miniCDX2 (lane 2) or the empty vector pFlag-CMV2 (lane 3) were analyzed by western blot with Flag, CDX2 or C2T antibodies. The Flag antibody reveals both Flag-CDX2 and Flag-miniCDX2 proteins, whereas the CDX2 and C2T antibodies, respectively, detect Flag-CDX2 and Flag-miniCDX2. (b) HCT116 cells co-transfected with pFlag-miniCDX2 and pEGFP showed nuclear localization of Flag-miniCDX2 (Flag antibody, red). Bars: 10 μ m. (c) Validation of the C2T antibody in mouse intestinal sections: nuclear immunostaining in the crypt epithelium of wild-type mice; loss of staining when C2T is preincubated with an excess of immunogenic peptide; absence of immunostaining in the intestinal epithelium of conditional knockout (KO) *Cdx2^{fl/fl}::AhCre^{ERT}* mice.⁷ Bars: 50 μ m. (C) Immunostaining pattern of the miniCDX2 (C2T antibody) and CDX2 proteins in the small intestine of E15 mouse embryos, and of post-natal day 2 (P2) and day 11 (P11) suckling and adult (Ad) mice. (b) Co-immunofluorescence detection of the miniCDX2 (C2T) and CDX2 proteins in the small intestine of adult mice. Bars: 100 μ m. (c) Immunostaining of the miniCDX2 (C2T) and CDX2 proteins in the proximal colon of adult mice. Bars: 50 μ m. Higher magnification pictures are given in the Supplementary Figure S3A. (D) MiniCDX2 expression in tumors of the proximal ileum of *Apc^{Δ14/+}* mice. (a) HE staining and C2T and CDX2 immunostaining. The region boxed in HE is shown in the immunohistochemistry pictures. Bars: 100 μ m. (b) RT-qPCR quantification of *CDX2* (light gray) and *miniCDX2* mRNA (dark gray) (semi-logarithmic scale) in wild-type ileum (wt), in three ileal tumors of *Apc^{Δ14/+}* mice (*Apc* T1-3) and in the adjacent normal mucosa (*Apc* N1-3); data are related to the amount of *CDX2* mRNA arbitrary set at 1 in the wild-type ileum. (c) Ratio between the *CDX2* and *miniCDX2* transcripts

This was accompanied by a higher expression of *PDX1*, an important regulator of BG morphogenesis²⁹ (Figure 5D) without change in cell proliferation and differentiation, as assessed by the absence of significant phospho-histone-3 labeling, and by the staining of neutral mucins (Figure 5E).

To address the consequence of the overgrowth of the BGs, mice were left to age until 18–24 months ($n = 19$). Fifty-two percent (10/19) of *jojo-Flag-miniCDX2::VilCre* mice developed one to two polyps in the pyloric region (Figure 6A), in contrast to control *jojo-Flag-miniCDX2* animals (1/12). These polyps

exhibited low- to high-grade dysplasia, without sign of neoplasia. As illustrated in Figures 6A and B, they were formed by expanded BGs extending even under the gastric mucosa, covered by disorganized crypts from the overlying duodenal epithelium, and by a surface epithelium with foregut-type properties, exhibiting gastric Claudin-18 expression and occasionally P63-positive squamous stratified epithelium. Phospho-histone-3 proliferating cells concentrated in the

disorganized crypt area. Consistent with the absence of neoplastic area, there was no evidence of β -catenin cytoplasmic/nuclear translocation. BGs produce EGF while adenomas develop after deletion of the EGF-receptor inhibitor *Lrig1*.³⁰ RT-qPCR revealed a 4–18 times higher level of EGF mRNA in the polyps of *jojo-Flag-miniCDX2::VilCre* mice compared to the duodenal mucosa of control animals ($n=3$). Linked to that, the disorganized crypt area showed elevated levels of

Figure 4 Molecular functions of miniCDX2 compared to CDX2. (A) Effect of miniCDX2 on the transcriptional activity of the CDX2 protein. (a) RT-qPCR quantification of the *Sl* RNA and (b) pSI-luc luciferase activity in HCT116 cells transfected either with pFlag-miniCDX2 or pFlag-CDX2 or pFlag-CDX2 together with increasing amounts of pFlag-miniCDX2; data are given as mean $\pm$ S.D. for triplicates (** $P<0.001$). (c) ChIP with anti-Flag or IgG in HCT116 cells transfected with pFlag-CMV2 (lane 1), pFlag-CDX2 (lane 2) or pFlag-miniCDX2 (lane 3); co-precipitated DNA was PCR-amplified with primers of the *Sl*, *LI-cadherin* and *Muc2* promoters. (d) ChIP with anti-CDX2 or IgG in HCT116 cells transfected either with pFlag-CMV2 (lane 1) or pFlag-CDX2 (lane 2) or pFlag-CDX2 together with a threefold molar excess of pFlag-miniCDX2 (lane 3) or pFlag-miniCDX2 (lane 4); co-precipitated DNA was PCR-amplified with primers of the *Sl* promoter. (B) Effect on miniCDX2 on β -catenin/Tcf4 signaling. (a) RT-qPCR quantification of the *Mmp7* RNA and (b) TOP-Flash luciferase activity in HEK293 cells transfected with pS33A- β -catenin, pMyc-Tcf4, pFlag-CDX2 and/or pFlag-miniCDX2; data are given as mean $\pm$ S.D. for triplicates (** $P<0.001$). (c) Proteins from cells transfected either with pS33A- β -catenin alone (lane 1), or pS33A- β -catenin together with pFlag-CDX2 (lane 2), or pS33A- β -catenin together with pFlag-miniCDX2 (lane 3) were immunoprecipitated with anti- β -catenin or IgG, and analyzed by western blot with anti- β -catenin and with anti-Flag to reveal Flag-CDX2 ($\triangleright$) and Flag-miniCDX2 ($\blacktriangleright$). (d) Proteins from cells transfected with pS33A- β -catenin and pMyc-Tcf4 together with increasing amounts of pFlag-CDX2 or pFlag-miniCDX2 were immunoprecipitated with anti- β -catenin, and analyzed by western blot with anti- β -catenin and anti-Myc to check the β -catenin/Tcf4 interaction. (C) Effect of miniCDX2 on P27^{KIP1}. (a) Representative western blot and quantification and (b) RT-qPCR quantification of P27^{KIP1} protein and RNA in HCT116 cells transfected either with pFlag-CMV2 (*ctrl*) or pFlag-CDX2 or pFlag-miniCDX2. (c) Representative pictures and quantification of clonogenic assays on HCT116 cells transfected either with pFlag-CMV2 (*ctrl*) or pFlag-CDX2 or pFlag-miniCDX2. Data are means $\pm$ S.D. for triplicates (** $P<0.01$). (D) Effect of miniCDX2 on double-strand break DNA repair. (a) Proteins from cells transfected either with pFlag-CMV2 (lane 1), pFlag-CDX2 (lane 2) or pFlag-miniCDX2 (lane 3) were immunoprecipitated with anti-Flag or IgG, and analyzed by western blot with anti-Flag and anti-KU70/80 to check the interaction of Flag-CDX2 ($\triangleright$) or Flag-miniCDX2 ($\blacktriangleright$) with the KU70/80 complex. (b) For DNA repair assays, linearized plasmid pcDNA3 (lane 1) was incubated with nuclear extracts of SW480 cells transfected with pFlag-CMV2 (lane 2), pFlag-CDX2 (lane 3) or pFlag-miniCDX2 (lane 4), and then separated by electrophoresis on an agarose gel. The linear and repaired (circular and/or concatemers) plasmid forms are indicated

Figure 6 Duodenal lesions in aged *jojo-Flag-miniCDX2::VilCre* mice. **(A)** (a) Macroscopic view of two adjacent polyps grown in the duodenal region of a 18-month-old *jojo-Flag-miniCDX2::VilCre* mouse. (b) Histology of a polyp near the gastric-intestinal boundary (arrow); (i), (ii) and (iii), respectively, designate the expanded territory of Brunner's glands (encircled), the hyper-proliferating crypts and the surface area. The arrowhead locates squamous stratified epithelium. Bar: 500 μm. **(B)** Immunostaining of Flag-miniCDX2, phospho-histone-3, Claudin-18 and β-catenin in the regions (i), (ii) and (iii) of the duodenal polyp. Bars: 50 μm. **(C)** Phospho-EGFR immunodetection in the regions (i) and (ii) of the polyps, and in normal adjacent duodenal crypts. Bars: 50 μm

membranous phospho-EGF-receptor, as compared to normal adjacent duodenal crypts (Figure 6C). Thus, the miniCDX2-driven expansion of BGs leads to the overproduction of EGF that activates the EGFR and makes hyperproliferate the overlying duodenal crypts, ultimately forming polyps.

Autoregulation of the *CDX2* pre-mRNA splicing. *Cdx2*^{+/-} mice develop cecal heteroplasias exhibiting a shift toward a foregut-type differentiation.³ These lesions are devoid of CDX2 protein even though one gene copy is preserved. Since miniCDX2 overexpression also induces foregut-type transdifferentiation, this prompted us to look for miniCDX2 in the heteroplasias of *Cdx2*^{+/-} mice. Unlike CDX2 present in

the normal cecal epithelium but absent in the heteroplastic glands, miniCDX2 was detected not only in the normal epithelium but also in the heteroplastic structures, indicating a splicing shift in favor of *miniCDX2* in the heteroplasias (Figure 7a). This opens the possibility that the balance between both splicing forms might be regulated and depend on the CDX2 protein itself.

To investigate the mechanism of pre-mRNA splicing, we constructed the plasmid eC211 overlapping the exon-1, intron-1 and the fused exon-2/-3 of the *CDX2* gene, and having a Flag sequence preceding the Stop codon in exon-3 (Figure 7b). In HCT116 cells, eC211 encoded both CDX2-Flag and miniCDX2-Flag variants identified using Flag, CDX2

Figure 7 Autoregulation of the *CDX2* pre-mRNA splicing. (a) Immunodetection of the CDX2 and miniCDX2 (C2T antibody) proteins at the border between normal cecal epithelium and heteroplasia (asterisk) in *Cdx2*^{+/-} mice. Bars: 50 μ m. (b) Map of the eC2I1 reporter plasmid. (c) Production of the two protein variants CDX2-Flag (▷) and miniCDX2-Flag (◀) from the reporter plasmid eC2I1. HCT116 cells were transfected either with the plasmids eC2I1, pFlag-CDX2 or pFlag-miniCDX2, and the proteins were revealed using anti-Flag, anti-CDX2 and anti-miniCDX2 (C2T) antibodies, respectively. (d) HCT116 cells transfected with the plasmid eC2I1 or with the mutant form eC2I1m. (e) Inverse correlation between the production of miniCDX2-Flag and the endogenous level of CDX2 in HCT116 and SW480 colon cancer cell lines. Upper panel: HCT116 and SW480 transfected with eC2I1. Lower panel: endogenous level of expression of CDX2 protein in HCT116 and SW480 cells related to actin. (f) HCT116 cells transfected with eC2I1 and either the control empty plasmid, or the plasmids encoding pHA-CDX2 or pHA-miniCDX2. The lower panel confirms the expression of HA-CDX2 and HA-miniCDX2 in these cells, using anti-HA antibody

and C2T antibodies (Figure 7c). Mutating the GT alternative splicing donor site into GA to give eC2I1m compromised the production of miniCDX2-Flag (Figure 7d). Thus, eC2I1 represents an appropriate reporter system of the alternative splicing of the *CDX2* pre-mRNA.

Interestingly, transfecting eC2I1 in HCT116 and SW480 cells produced different rates of miniCDX2-Flag protein, inversely related to the respective endogenous level of CDX2 in these cells (Figure 7e). To address a possible role of the CDX2 protein in regulating its pre-mRNA splicing, HCT116 cells were co-transfected with eC2I1 and the plasmid encoding HA-CDX2. HA-CDX2 reduced the amount of miniCDX2-Flag produced by eC2I1, and this effect was also recapitulated by the transcription-deficient protein HA-miniCDX2 (Figure 7f). Thus, the CDX2 and miniCDX2 proteins exert a similar inhibitory effect on the alternative splicing leading to the *miniCDX2* variant, through a transcription-independent mechanism.

The sequence around the alternative splicing donor site of the *CDX2* pre-mRNA revealed several putative splicing factors

binding elements conserved in human and mouse (Supplementary Figure 4), in particular for the SRp30c and ASF/SF2 factors expressed in intestinal cell lines (Figure 8A). In transfected HCT116 cells, His-ASF/SF2 inhibited the splicing leading to miniCDX2-Flag, whereas His-SRp30c stimulated it; in addition, the stimulatory effect exerted by His-SRp30c was abolished by His-ASF/SF2 (Figure 8Ba). Of note, CDX2 also counteracted the effect of SRp30c (Figure 8Bb). Co-immunoprecipitation (coIP) was performed to investigate possible interactions between these factors. In transfected HCT116 cells, His-SRp30c co-immunoprecipitated with His-ASF/SF2 (Figure 8Ca). A similar result was obtained with the endogenous SRp30c and ASF/SF2 proteins, and the amount of co-precipitated material increased by RNase treatment, indicating an RNA-independent interaction (Figure 8Cb). Interestingly, HA-CDX2 also interacted with the endogenous ASF/SF2 (Figure 8Da) and SRp30c proteins (Figure 8Ea), and the amount of complex increased by RNase treatment. HA-miniCDX2 gave the same results, showing that the transactivation domain and activity of CDX2 is dispensable

Figure 8 Mechanism of regulation of the *CDX2* pre-mRNA splicing. (A) Expression of the splicing factors ASF/SF2 and SRp30c in HCT116 and SW480 colon cancer cells. (B) Impact of ASF/SF2, SRp30c and CDX2 on the reporter plasmid eC211. HCT116 cells were transfected with eC211 and the expression plasmids for the indicated proteins, and the two splicing variants CDX2-Flag (▷) and miniCDX2-Flag (▶) were detected with anti-Flag antibody. (C) Interaction between ASF/SF2 and SRp30c. (a) HCT116 cells were transfected with the plasmids encoding His-ASF/SF2 and His-SRp30c. After immunoprecipitation with anti-ASF/SF2 antibody or with IgG, the proteins were revealed with anti-ASF/SF2 and anti-SRp30c antibodies. (b) Endogenous proteins of HCT116 cells were immunoprecipitated with IgG or with anti-ASF/SF2 antibody without or with pre-treatment with RNase, and revealed with anti-SRp30c antibody. (D) Interaction between ASF/SF2 and CDX2 or miniCDX2. (a) HCT116 cells were transfected with the control plasmids pCMV2-Flag (lane 1) or pHA-CDX2 (lane 2). Protein extracts immunoprecipitated with anti-HA beads, and revealed with anti-HA and anti-ASF/SF2 antibodies. (b) Same as (a) with pHA-miniCDX2 instead of pHA-CDX2. (E) Interaction between SRp30c and CDX2 or miniCDX2. Same as (D) except that protein detection used anti-SRp30c instead of anti-ASF/SF2 antibody. (F) Effect of CDX2 and miniCDX2 on the interaction between ASF/SF2 and SRp30c. (a) HCT116 cells were transfected with the control plasmid pCMV2-Flag (lane 1), with pHA-CDX2 (lane 2) or pHA-miniCDX2 (lane 3). Cell extracts were immunoprecipitated with anti-ASF/SF2 antibody, and revealed with anti-ASF/SF2 and anti-SRp30c antibodies. (b) Quantification of immunoprecipitated SRp30c relative to ASF/SF2

for its interaction with splicing factors (Figures 8Db). Hence, we investigated the impact of CDX2 and miniCDX2 on the ASF/SF2 / SRp30c complex. For this purpose, cells were transfected either with the control plasmid or pHA-CDX2 or pHA-miniCDX2, and nuclear extracts were processed for immunoprecipitation with anti-ASF/SF2 antibody. As shown in Figure 8F, the amount of SRp39c co-immunoprecipitated with ASF/SF2 increased in the presence of HA-CDX2 or HA-miniCDX2, respectively, by 1.7- and 1.6-fold compared to the control. Thus, CDX2 and miniCDX2 stimulate the SRp30c/ASF/SF2 interaction, which subsequently hampers the selection of the alternative splicing donor site used to produce the *miniCDX2* splicing variant.

Discussion

This work reports a number of novel exon–exon junctions for genes expressed in the gut, which deserves future studies of the relevance of RNA splicing in intestinal development, homeostasis and disease. Focusing on the homeobox gene *CDX2*, a major regulator of gut ontogenesis and function,^{4,8,28} we identified the miniCDX2 variant, resulting from the combination of alternative pre-mRNA splicing and alternative translation initiation. This variant fine-tunes CDX2 activity through versatile functions regarding the full-length homeoprotein: miniCDX2 antagonizes the transcriptional activity of CDX2 by DNA-binding competition, it is neutral with respect to the inhibitory function exerted by CDX2 on β -catenin/Tcf4 signaling, and it has the same activities as CDX2 on cell proliferation, DNA repair and RNA splicing through P27, KU70/80 and splicing factors, respectively. The production of miniCDX2 is based on a conserved sequence motif among *CDX2* paralog in vertebrates, that is absent in other members of the caudal family, with the exception of *Cdx1b* in teleost fishes. Interestingly, in teleosts, the *CDX2*-type gene has been lost and replaced by a duplicated version of *CDX1* (*Cdx1b* in *D. rerio*), which is functionally equivalent to the mammalian *CDX2* gene for intestinal development.³¹ Thus, *Cdx1b*, unlike *Cdx1a*, may have acquired a similar motif as *CDX2*, although being a gene of the *CDX1* type. The conserved motif of the *CDX2*-type genes has implications for translation initiation. Indeed, in the context of the *CDX2* mRNA, the AUG₂ codon used for CDX2 translation is preceded by the upstream and out-of-frame AUG₁ that could potentially initiate the synthesis of a 13-aa peptide. Although the existence of this peptide remains elusive, short upstream open reading frames are relatively frequent and can control the translation of the downstream open reading frame or even be involved in diseases.³²

In the gut, both the loss or the excessive expression of CDX2 are lethal,^{8,9} highlighting the need for a precise regulation of the activity of the gene. Previous studies have already reported transcriptional^{33,34} and post-translational levels of regulation.^{35,36} This study adds a novel post-transcriptional level of regulation through the production of the variant miniCDX2 that fine-tunes the activity of CDX2. Another variant of CDX2 has been reported, CDX2-A/S, described as a regulator of splicing.³⁷ The present study further uncovers that the transcription factor CDX2 and its variant miniCDX2 also act as splicing regulators through a

non-transcriptional mechanism involving their interaction with splicing factors. This involvement in pre-mRNA splicing might considerably broaden the sphere of activity of the *CDX2* homeobox gene. Interestingly, miniCDX2 is expressed in the crypts, like the S60-phosphorylated form of CDX2 characterized by a reduced transcriptional activity.³⁶ This suggests that combined mechanisms act to prevent excessive activity of the CDX2 homeoprotein in stem/progenitor cells and hence the premature cell differentiation known to result from excessive CDX2 in the crypts.⁹

CDX2 has a role in the patterning of the intestine.³⁸ Here, overexpressing miniCDX2 leads to extend the BG territory, which is accompanied by an increase of *PDX1*, a homeobox gene of the *paraHox* cluster, like *CDX2*, involved in BG morphogenesis.²⁹ This suggests that PDX1, together with the CDX2/miniCDX2 balance, participate in shaping the gastric-intestinal boundary, in that the BG territory would depend on a low activity of CDX2 in the proximal small intestine, whereas miniCDX2 overexpression, through DNA-binding competition with CDX2, would allow extending the territory of these glands. In the long term, miniCDX2 overexpression in the BGs leads to brunneroma in which the production of EGF is increased and activates the EGFR in the overlaying crypts. Interestingly, these polyps exhibit gastric-type differentiation and even squamous stratified differentiation, but do not spontaneously progress in cancer, like most brunneroma in man. In line with this, the foregut-type heteroplasia developing in the cecum of *Cdx2*^{+/-} mice³ and the cecal gastric-type lesions generated by conditional *Cdx2* knockout in the adult gut²⁸ also fail to spontaneously progress to malignancy. Thus, the overexpression of the dominant-negative variant miniCDX2 and the loss of function of CDX2 do not represent oncogenic events *per se*. Yet, CDX2 has a tumor suppressor activity in the gut, as assessed by model studies in mice^{12,13} and observations made in colon cancer patients.^{10,39} Here, the data showing a modified CDX2/miniCDX2 ratio in favor of miniCDX2 in intestinal cancer open the possibility that miniCDX2, through its dominant-negative effect exerted on the tumor suppressor CDX2, may contribute to disease progression.

Here, we uncovered and characterized an original variant of the *CDX2* homeobox gene, which is a major player of intestinal morphogenesis, homeostasis and also involved in gut cancer. The production of this variant represents not only a novel level of regulation of this gene, but also a new way to fine-tune its biological activity through the versatile functions exerted by the truncated variant compared to the full-length homeoprotein. As in the case of another important transcription factor, SOX9,⁴⁰ this study highlights the relevance of generating protein diversity through alternative splicing in the gut and its diseases.

Materials and Methods

Mice and generation of transgenic animals. Mice were handled according to the protocol approved by the Committee on the Ethics of Animal Experiments of the University of Strasbourg (CREMEAS, C2EA-35) under the permit number AL/43/50/02/13.

To generate *jojo-Flag-miniCDX2* mice, the Flag-miniCDX2 sequence of plasmid pFlag-miniCDX2 (see below) was PCR-amplified using the primers AAAAGTC GACTACCATGGACTACAAAGACGATGA/AAAAGTCGACTCAGCCTGGAATTGCTC TGCCG containing each a Sall restriction site. The resulting Sall fragment was inserted in the XhoI site of the vector jojo⁴¹ to get plasmid jojo-Flag-miniCDX2. The

eluted 8010 bp *Sall* fragment containing the transgene was injected in mouse eggs at the Mouse Clinic Institute (Illkirch, France). Mice were PCR-genotyped with primers 39/hC2E3R (AGTCATAGCTGTCCTCTTC/TCAGCCTGGAATTGCTCTGC).

Cdx2^{-/-}, *VilCre*⁴³ and *Apc*^{Δ14/+} mice⁴⁴ have been described. *Sox2Cre* mice were obtained from The Jackson Laboratory (Charles River, L'Arbresle, France).

Cell lines and colony formation assays. Human colon cancer cell lines Caco2TC7,⁴⁵ HCT116,⁴⁶ SW480⁴⁷ and T84,⁴⁸ and human embryonic kidney cells HEK293⁴⁹ were grown as recommended. Colony formation assays were performed on HCT116 cells transfected with the indicated plasmids, as previously described.³⁵

Plasmids

pFlag-CDX2 and pFlag-miniCDX2. RNA extracted from Caco2TC7 cells was subjected to RT-PCR using Platinum TaqPCRx DNA polymerase (Promega Inc., Madison, WI, USA) with the primers AAAAAGCTTTACGTGAGCTACCTCTGGACAAGG and AAAATCTAGATCAGCCTGGAATTGCTCTGC to amplify the human CDX2 coding sequence, and with the primers AAAAAGCTTGTGAGGTC TGCTCCCGGACCCTCGC and AAAATCTAGATCAGCCTGGAATTGCTCTGC to amplify the miniCDX2 coding sequence. PCR fragments were inserted in pCRII-TOPO (TA cloning kit, Life Technologies Invitrogen, Villebon sur Yvette, France), giving, respectively, pCRII-TOPO-CDX2 and pCRII-TOPO-miniCDX2. These plasmids were cut with *HindIII* and the fragments inserted in the *HindIII* site of pFlag-CMV2 (Sigma-Aldrich, St. Louis, MO, USA) to get the plasmids pFlag-CDX2 and pFlag-miniCDX2, respectively.

pATG1-ATG2 and pATG1m-ATG2. To construct pATG1-ATG2, the 5'-untranslated region of the mouse *Cdx2* gene up to the codon ATG₂ was PCR-amplified from pSL1190-9.47³³ with the primers TTTTAAGCTTAAGGCCGCTGGC CTGGGACTCCGCGA/TTTTCATGGTGGCGAGGACCCAGAGCAGA containing, respectively, *HindIII* and *NcoI* sites. The PCR fragment was cut with *HindIII* and *NcoI*, and inserted in the corresponding sites of pGI3-control (Promega Inc.) to give pATG1-ATG2. pATG1m-ATG2 was derived from pATG1-ATG2 by site-directed mutagenesis using the Quick Change IIXL Site Directed Mutagenesis Kit (BD Biosciences, San Jose, CA, USA) with the primer TCAACGTTTGTCCCGAGACA CCTAGGTGAGGTCTGCTCTGGGT to change the ATG₁ start codon into TAG.

pATG1 and pATG1m. Plasmid pATG1 containing the 5'-untranslated region of the murine *Cdx2* gene up to the codon ATG₁ was constructed as above by PCR amplification with the primers TTTTAAGCTTAAGGCCGCTGGCCTGGGACTCCG CGA and TTTTCATGGTGTCTGGGACAAACGTTGT in which the sequence was changed for one nucleotide to create a *NcoI* cloning site. After insertion of the PCR fragment in the *HindIII* and *NcoI* sites of pGI3-control, the correct sequence was restored by point mutagenesis with the primer TTTGTCCCCAGACAGCATGG AAGACGCCAAAACAT to give pATG1. pATG1m was derived from pATG1 by mutation with the primer TTTGTCCCCAGACAGCATAGGAAGACGCCAAAACAT to change the ATG₁ start codon into TAG.

eC21l and eC21lm. The human *CDX2* minigene overlapping the exon-1, the intron-1 and the fused exon-2/3 and having the Flag coding sequence inserted before the Stop codon in the exon-3 was sequentially constructed using three overlapping fragments generated by PCR using Platinum TaqPCRx DNA Polymerase (Life Technologies Invitrogen): the fragment A and B were, respectively, amplified by PCR from human genomic DNA with the primers Af (GTTTA AACTTAAGCTTGCTCCGACGCCAGCCTGT)/Ar (TCACAGGTCAGAGGTTTCAGAG), and with the primers Bf (CTCTGAACCTCTGACCTGTGA)/Br (TCCGTGTACAC CACTCGATATT); the fragment C was amplified from pFlag-CDX2 DNA using the primers Cf (AGTGGGTGACACGGACCACC) and Cr (GCCCTCTAGACTCGAGTC ACTTGTCTGTCATCGCTTTGTAGTCTGGGTGACGGTGGGGTTTA). Af contains 15 nucleotides complementary to the cloning site of the vector pcDNA4/TO, followed by 20 nucleotides complementary to the 5' extremity of the *CDX2* gene; Cr contains 15 nucleotides complementary to the cloning site of pcDNA4/TO, followed by a translation Stop codon, 24 nucleotides complementary to the Flag sequence and 20 nucleotides complementary to the *CDX2* gene; the primers Ar/Bf are partially complementary over 15 nucleotides, as the primers Br/Cf. The three PCR fragments A, B and C and the vector pcDNA4/TO cut with *HindIII* and *XhoI* were purified by agarose gel electrophoresis, mixed and incubated with the In-Fusion HD Cloning kit (Clontech Laboratories, Inc., Mountain View, CA, USA). The resulting plasmid eC21l was verified by sequencing. eC21lm was derived from eC21l using the primer ACCCTCGCCACCATGAACGTGAGCTACCTCC to change the GT splicing site into GA.

Other plasmids were: pS33A- β -catenin encoding activated β -catenin,⁵⁰ pcDNA3-Tcf4E-myc encoding Myc-tagged Tcf4,⁵¹ the reporter plasmids TOPFlash,⁵²

pSI-Luc,⁵³ pHIS-SRp30c and pHIS-ASF/SF2,⁵⁴ and commercially-available pEGFP (Clontech Laboratories Inc.), pGI3-basic, pGI3-control, pRL-Null (Promega Inc.) and pCMV-Flag2 (Sigma-Aldrich).

Antibodies. The antibody against the miniCDX2 protein was obtained by immunizing rabbits with the synthetic peptide MVRAPGPSPP-cysteine coupled to KLH (Eurogentech SA, Seraing, Belgium). C2T anti-peptide antibody was purified by affinity chromatography. The peptide was reduced by TCEP (Tris-(2-carboxyethyl) phosphine) using Immobilized TCEP-Disulfide Reducing gel (Pierce Biotechnology, Rockford, IL, USA). Reduced peptide of 2.5 mg were incubated with 2 ml sulfonyl-agarose (SulfoLink Trial kit, Pierce Biotechnology) during 1 h and the affinity gel was equilibrated in Tris 50 mM/NaCl 0.15 M pH 7.3 buffer. Rabbit immune serum of 2 ml were passed through the affinity column, washed with Tris 50 mM/NaCl 0.15–0.5 M pH 7.3. Specific immunoglobulins were eluted with glycine 0.2 M pH 2.7 and neutralized with 50 μ l Tris buffer 1 M pH 8. The C2T antibody raised against the Nter part of miniCDX2 was used at 15 μ g/ml.

Commercial antibodies were against: actin (clone C4, Millipore, St. Quentin-en-Yvelines, France), ASF/SF2 (sc-33652, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), β -catenin (mouse clone 14, BD Transduction Lab, San Jose, CA, USA), CDX2 (mouse clone 392M, BioGenex, San Ramon, CA, USA), Claudin-18 (Life Technologies Invitrogen), Flag (mouse clone M2, Sigma-Aldrich), GFP (BD Biosciences, Franklin Lakes, NJ, USA), HA (clone 3F10, Roche Applied Science, Meylan, France), KU70 (rabbit clone EPR4027, Abcam, Cambridge, MA, USA), KU80 (rabbit clone EPR3468, Abcam), Myc (mouse clone 9E10, Santa Cruz Biotechnology Inc.), p27KIP1 (mouse clone 57, BD Transduction Lab), PDX1 (rabbit, Abcam ab47267), phospho-EGFR (Cell Signaling Technology, Ozyme, Montigny-le Bretonneux, France), phospho-histone-3 (Ser10 mitosis marker, Millipore Upstate, Saint-Quentin Yvelines, France) and SRp30c (sc-134046, Santa Cruz Biotechnology Inc.).

Cellular RNA preparation and *in vitro* RNA transcription. RNA was prepared using TRI Reagent (Life Technologies Ambio, Villebon sur Yvette, France) and treated with DNase (RQ1 RNase-free DNase, Promega Inc.).

For *in vitro* transcription of capped and uncapped RNA, DNA templates were amplified using Platinum TaqPCRx DNA polymerase from the plasmids pATG1, pATG1-ATG2, pATG1m-ATG2 and pGL3-control with T7/T7pion-r (TTTTTTTTTT TTAATACGACTCACTATAGGAGAAAGCCATAGGCTTTTGCAAAAAGCTT/T)₄₂CACT GCATCTAGTTGTG) containing, respectively, the T7 promoter and polydT sequences. After purification (QIAquick PCR Purification kit, Qiagen, Venio, The Netherlands), 1 μ g of each PCR fragment was *in vitro* transcribed with or without m7G(5')ppp(5')G RNA Cap (S1404S, BioLabs, Ipswich, MA, USA) in the ratio of 1 GTP/4 GTPcap, in 25 μ l using HiScribe T7 *in vitro* Transcription kit (BioLabs). After treatment with DNase (RQ1 RNase-free DNase, Promega Inc.), RNAs were precipitated in NH₄-acetate 2.5 M on ice, washed in 70% ethanol and solubilized in 5 μ l RNase-free water.

RNAseq analysis. RNA integrity was checked on nano RNA chips with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and no sign of RNA degradation was detected. Total RNA of 1 μ g was used to construct the mRNAseq libraries with Illumina's TruSeq RNA sample kit following manufacturer's instructions (Illumina, San Diego, CA, USA). Libraries were validated and quantified on DNA1000 chips and a Bioanalyzer 2100 (Agilent Technologies, Les Ulis, France). Paired-end reads of 50 bp were obtained with a HiSeq1000 by multiplexing three libraries on one lane. Demultiplexing and generation of raw fastq files were performed with CASAVA v1.7. Mapping against the reference mouse genome GRCm38 was performed with tophat 2.0.11 and bowtie2 using the options -b2-sensitive -a 5 -p 5 -library-type fr-unstranded -r 180 -mate-std-dev 80. Quantification of the reads was performed with HTSeq and DESeq2 using the reference gene annotation from ensembl v75. Genes with > 10 normalized reads in the three replicates as given in DESeq2 were considered as significantly detected. *De novo* transcripts assembly was performed with cufflinks v2.2.1 with the options -u -library-type fr-firststrand, and providing the reference genome fasta file and gene annotation from ensembl v75. Search of novel exon-exon junctions was made with cuffcompare and consensus novel junctions found in all biological triplicates obtained with bedtools. The resulting bed file was then imported in R as a Grange object for gene annotation.

Northern blots, RT-PCR and RT-qPCR. PolyA+ RNA (PolyAtract System, Promega Inc.) was separated by electrophoresis on 2% agarose/2 M formaldehyde

gels (10 µg polyadenylated RNA/lane) and transferred to nylon membranes. Hybridization and revelation was performed as described⁵⁵ with 100 ng/ml of DIG-labeled antisense cRNA probe. This probe overlapped the 3'-untranslated region of the exon-3 of the human *CDX2* gene, and was *in vitro* transcribed by T7 RNA polymerase from plasmid pCRLI-TOPO-miniCDX2 linearized with BsrGI. A DIG-labeled human GAPDH probe was used for normalization.

Reverse transcription was performed with 2 µg RNA using SuperScript II Reverse Transcriptase (Life Technologies, Invitrogen) and Oligo(dT)12-18 primer (Invitrogen).

RT-PCR used 1/10th of the RT solution. Concomitant amplification of the two splicing forms of the human *CDX2* gene was performed with primers CDX21F/CCR (GCAGCCTCCAGCGTCGGTC/TCAGCCTGGAATTGCTCTGC) for 35 cycles (30 s at 95 °C, 30 s at 55 °C and 1.5 min at 68 °C). Specific amplification of the mouse *CDX2* mRNA and *miniCDX2* mRNA, respectively, used the primer pairs mhC2FII/mhC2E3r (CCCTCGCCACCATTGACG/CTCTCGCGTTCTGAAACCA) and mhC2Tqf (CCCTCGCCACCATTGAAAA)/mhC2E3r.

For RT-qPCR, 2 µg of mouse intestinal RNA, of RNA from human colon biopsies or from human colon cancer Caco2TC7 cells were reverse transcribed in 20 µl using the AMV Reverse Transcriptase kit (Life Technologies, Invitrogen) and oligo-dT. RT-qPCR was performed on 1 µl of RT reaction in 20 µl using Syber Green PCR Master Mix (Life Technologies Applied Biosystems, Villebon sur Yvette, France) and 7500 Real-time PCR System (Life Technologies Applied Biosystems). For mouse RNA, the cycles were 95 °C 15 s, 57 °C 1 min, with the primers: CCCTCGCCACCATTGAAAA/GTGATGTATCGACTAAAGTG for *miniCDX2*, CTAGGAAGCCAAGTGAAAC/GTGATGTATCGACTAAAGTG for *CDX2* and CCCACAACTCTTCCATTCT/GCAGGAGTGATAGGGGTCAT for *TBP*. For Caco2TC7 cell RNA, the cycles were 95 °C 15 s, 68 °C 1 min with the primers: CCCTCGCCACCATTGAAAA/GCCTCTCAGAGAGCCCCAGCGTGG for *miniCDX2*, CTCGGCAGCCAAGTGAAAC/GTGATGTAGGACTGTAGTG for *CDX2* and TGCACAGGAGCCAAGAGTGAA/CACATCACAGCTCCCCACCA for *TBP*.

Quantification of the *Sl*, *MMP7*, *P27KIP1*, *EGF* and *TBP* transcripts was performed by RT-qPCR using diluted RT products (5–10 ×) mixed with TaqMan Master Mix, gene-specific TaqMan probe and the primers sets (TaqMan Gene Expression assays, Life Technologies Applied Biosystems): *Sl*, Hs00356112_m1; *MMP7*, Hs01042795_m1; *P27KIP1*, Hs01597588_m1; *EGF*, Hs01099999_m1; *TBP*, Hs99999910_m1.

Analysis of the results (triplicates) was performed with the 7500 software v2.0.1 (Life Technologies Applied Biosystems) using the relative $\Delta\Delta C_t$ quantification method.

DNA and RNA transfections and luciferase assays. Plasmid DNA transfection was performed during 48 h with JetPEI (Polyplus Transfection, Illkirch, France). RNA transfection was performed for 3 h with 100–500 ng of *in vitro*-transcribed RNA (see below) using 0.25–1.25 µl TransIT mRNA and 0.375–1.875 µl of boost (Mirus Bio, Madison, WI, USA) on cells at 80% confluence. Firefly and Renilla luciferase activities were measured at least three times in triplicate using a dual reporter luciferase assay (Promega Inc.) in cell extracts prepared 48 h after DNA transfection or 3 h after RNA transfection. Means are given $\pm$ S.D.

Immunostaining. Immunohistochemical staining and/or immunofluorescence detection were performed on paraffin-embedded tissue sections.³³ Histochemical staining used the Vectastain ABC kit (Vector Labs, Peterborough, UK) and DAB 0.3 mg/ml/H₂O₂ 0.03%. Double-immunofluorescence staining used secondary Alexa488-labeled anti-mouse antibody and Alexa688-labeled anti-rabbit antibody (Life Technologies Invitrogen) coupled to fluorescence amplification with the TSA Fluorescent Plus System (Perkin Elmer, Waltham, MA, USA).

Co-immunoprecipitations and western blots. Transfected cells were lysed in buffer containing 50 mM Tris buffer pH 7.4, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). The buffer additionally contained 1% Triton X-100 for β -catenin and Flag immunoprecipitation, or 1% Triton X-100 and 1% NP-40 for ASF/SF2 immunoprecipitation, or 1% NP-40 for HA immunoprecipitation. Lysates were cleared by centrifugation at 12 000 × *g* for 20 min at 4 °C, and the protein extracts were then treated with 1 µl of 0.1 mg/ml RNaseA for 30 min on ice when indicated. Immunoprecipitation was performed using 1 mg of lysate supernatant incubated overnight at 4 °C with gentle rocking in 1 ml of lysis buffer with the appropriate antibody: anti-Flag antibody (5 µg of clone M2, Sigma-Aldrich), anti- β -catenin antibody (1 µg of clone 14, BD Biosciences), anti-HA antibody (20 µl of agarose beads coupled to clone 3F10, Roche Applied Science) and anti-ASF/SF2 antibody (3 µg of sc-33652, Santa Cruz Biotechnology Inc.). After adding 25 µl of Protein-G-agarose beads (Roche Applied

Science) for 2 h at 4 °C, the beads were washed in lysis buffer. The immunoprecipitated material was eluted using SDS-PAGE sample loading buffer and analyzed by western blots performed as described.³⁵

Chromatin immunoprecipitations. Transfected HCT116 cells were fixed with 1% (v/v) formaldehyde for 10 min at RT and quenched with 0.125 M glycine for 5 min. ChIPs were carried out using the EZ-Magna ChIP G Chromatin Immunoprecipitation kit (Upstate Millipore, Billerica, MA, USA). Sheared cross-linked chromatin from ~10⁶ cells was incubated overnight at 4 °C with 1 µg of normal mouse IgG (Upstate Millipore) or the appropriate antibody. Input corresponds to non-immunoprecipitated sheared cross-linked chromatin from 10⁵ cells. PCR analysis was performed with 1/25th of immunoprecipitated DNA as template. The primers used for PCR amplification of three human targets of the *CDX2* protein were: *Sl* promoter GGCTGTAAGGGTGCAATAA/GCCTGTTCTTTGCTATGTTG; *Muc2* promoter: GTGTGTTGGCATTGAGGCTA/AGGGTAGGAGGGCAGGAAG; *Li-cad* promoter: TTTCAAAAGCAGGCACAGCC/AGTGGTCGAGACTCTTGCTAC.

In vitro DNA repair assays. Cell-free extracts were prepared as follows. A total of 3 × 10⁷ transfected cells were washed in cold PBS, suspended in four packed cell volumes of cold hypotonic lysis buffer (10 mM Tris-HCl pH 7.6, 1 mM DTT, 5 mM MgCl₂, 1 mM EDTA and protease inhibitors), incubated for 40 min on ice and disrupted using a Dounce homogenizer (40 strokes). Sucrose was added to reach 250 mM. Extracts were centrifuged 10 min at 1000 × *g* to discard cell debris and washed again in hypotonic lysis buffer containing 250 mM sucrose. Nuclear pellet was then suspended in two volumes of nuclear extraction buffer (20 mM Tris-HCl pH 7.6, 1 mM DTT, 2 mM EDTA, 20% glycerol, 500 mM NaCl and protease inhibitors) and incubated for 30 min on ice. Nuclear extracts were clarified by 30 min centrifugation at 21 000 × *g* and dialyzed overnight against 20 mM Tris-HCl pH 7.6, 1 mM EDTA, 1 mM DTT, 20% glycerol, 25 mM NaCl and 0.2 mM PMSF.

End-joining reaction was performed in 30 µl by incubating 50 ng of BamHI-linearized pcDNA3 plasmid with 5 mg of nuclear extract for 1 h at 25 °C in 50 mM Tris-HCl pH 7.6, 5 mM MgCl₂, 1 mM ATP, 1 mM DTT, 50 mM dNTP, 80 mM NaCl and protease inhibitors. Reaction was stopped by RNase treatment (0.25 mg/ml of RNase for 10 min at 37 °C), followed by proteinase K (0.5% SDS, 50 mM EDTA and 1 mg/ml of proteinase K at 37 °C for 1 h). DNA was purified by phenol and chloroform extraction, recovered by ethanol precipitation and analyzed by electrophoresis on a 0.85% agarose gel.

Statistical analyses. Experiments were performed in at least three independent biological replicates in triplicates. *P*-value calculation indicated significant differences tested by the Wilcoxon–Mann–Whitney test.

Conflict of Interest

The authors declare no conflict of interest.

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